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Clonal Propagation of White Flowered Hybrid *Cattleya* through *in Vitro* Culture of Thin Cell Layers from Young Shoots of Mature Plants

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ABSTRACT

A protocol for the clonal propagation of *Cattleya* was developed from thin cell layers (TCLs) derived from young shoots through *in vitro* culture. The TCLs from young shoots of a mature *Cattleya* hybrid was cultured for one week on half strength Murashige and Skoog (MS) liquid medium supplemented with N^6-benzyladenine (1.5 mg l^{-1}), α-naphthaleneacetic acid (0.5 mg l^{-1}), 2% (w/v) sucrose, 10% (v/v) coconut water, 2 g l^{-1} peptone and 1 g l^{-1} activated charcoal, followed by culture on agar-gelled full strength MS medium with same supplementing components, which produced an average of 8 protocorm-like bodies (PLBs) within 12 weeks. Clumps of PLBs were subcultured for 8 weeks on fresh medium with the same nutrient composition and cut into eight pieces and subcultured on Gelrite-gelled MS medium with 2% (w/v) sucrose + 10% (v/v) coconut water + 2 g l^{-1} peptone + 150 mg l^{-1} L-glutamine + 1 g l^{-1} activated charcoal, where each clump fragment of the PLBs produced an average of 220 PLBs within 8 weeks. After a further four weeks of subculture the PLBs were found to have enlarged with leafy shoots and new PLBs induced from the base of the old leafy bodies. Plantlet development from leafy shoots was achieved on ½MS medium supplemented with 2 g l^{-1} peptone, 2% (w/v) sucrose, 10% (v/v) CW and 1 g l^{-1} activated charcoal, where 100% of explants developed into plantlets with roots within 8 weeks. The addition of 50 g l^{-1} banana pulp in the medium enhanced the number and length of roots. Within the first 41 weeks after initiation of culture 1760 plantlets as well as a huge amount of PLBs were induced from a single explant (TCL) section. Repeating the subculture of PLBs on proliferation medium and culturing leafy shoots on plantlet regeneration medium could produce 500,000 plantlets every 32 weeks.

Keywords: protocorm-like bodies, regeneration

INTRODUCTION

Several techniques have been developed for the clonal propagation of *Cattleya* through tissue culture (Ball *et al*. 1971; Bergman 1972; Churchill 1972; Ichihashi and Kako 1973; Arditti 1977; Huang 1984; Torres and Jimenez 2004). However, not all of these methods can be used for commercial micropropagation because of differences in survival rate, PLB formation, and plantlet regeneration. Moreover, considerable variability exists between media used for the propagation of even a single group of orchids (Huang 1984). Previous researchers used shoot, or bud meristems, leaf tips, back bulbs, etc. as explants for regeneration of *Cattleya*. As for its cut flowers, *Cattleya* is a very important crop and clones of disease-free, photoperiodically controllable varieties are well suited as cut flowers, are very desirable and can be profitable. Orchid tissue cultures contain four component groups: (i) minerals (macro and micro), (ii) an energy source (always a sugar, usually sucrose), (iii) substances like vitamins and hormones, (iv) undefined complex additives (coconut water, potato extract, banana homogenate, peptone, casein hydrolysate, etc.) and the most appropriate combination can be expected to produce the best result (Arditti and Ernst 1993). So, the media to be used to obtain the best result should have the most suitable components and explants. Recently thin cell layers (TCLs) made transversely (tTCLs) from either stem, leaf, root or inflorescence axis have also been used for regeneration of ornamental plants (Prakash *et al*. 1996; Bui *et al*. 1999; Nhut *et al*. 2001; Teixeira da Silva 2002; Nhut *et al*. 2006). tTCLs include a small number of cells from different tissue-types: epidermal, cortical, cambium, prevascular and medullar tissue, parenchyma cells (Tran Than Van 1980). The cells within the TCL may redifferentiate into organs exhibiting correct developmental patterns leading to phenotypically normal organ architecture (Teixeira da Silva and Nhut 2003). As there is no report on clonal propagation of *Cattleya* through *in vitro* culture of TCLs, we undertook this for quick high frequency regeneration. Thus this paper reports an efficient and quick method for repetitive high frequency clonal propagation of *Cattleya* through *in vitro* culture of TCL of young shoot tips from mature plants.

MATERIALS AND METHODS

Plant material and induction of protocorm-like bodies (PLBs)

A white cultivar of *Cattleya* hybrid was selected for an explant source. Shoot apex explants were excised from newly emerging shoots (2-3 cm long) of mature plants. The outer leaves were removed to expose the terminal portion of the stem. The explants were washed under running tap water followed by detergent, Tween 80 (5% v/v) for 5 min. After a thorough wash with double-distilled water, surface sterilization was done with a 0.1% (w/v) mercuric chloride solution for 8-10 min. The explants were then washed thoroughly with sterile double-distilled water. The surface sterilized explants were then prepared for inoculation. For direct induction of protocorm-like bodies (PLBs), the basal portion of the explants were cut into thin transverse sections (1.0-2.0 mm) manually. The explants were cultured in ½MS (Murashige and Skoog 1962) liquid medium for one week – one explant per sterile glass

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test tube (25 × 150 mm) containing 12 ml of medium. – and then cultured on gelled MS medium supplemented with N^6-benzyladenine (BA, 0.5-2.5 mg l^{-1}) and Kinetin (Kn, 0.5-2.5 mg l^{-1}) individually and in combination with α-naphthaleneacetic acid (NAA, 0.2-0.5 mg l^{-1}) along with 2% sucrose, 10% (v/v) coconut water (CW), 2 g l^{-1} peptone and 1 g l^{-1} activated charcoal (AC). The medium was gelled with 2.2 g l^{-1} Gelrite (Duchefa, The Netherlands). The pH of the media was adjusted to 5.6 before autoclaving at a pressure of 105 kPa for 20 min at 121°C. All cultures were incubated at 24 ± 1°C, under cool white fluorescent light of 30 μm $^{-2}$ s^{-1} for 16 h per day.

Proliferation of PLBs

For proliferation of PLBs and formation of shoots, the clump of PLBs developed from initial explants was subcultured in the same nutrient medium for 8 weeks and cut into 8 pieces (5-6 PLBs in each piece) and subcultured on two different media, MS and ½MS supplemented with 2% sucrose, 2 g l^{-1} peptone, 1 g l^{-1} AC and with or without CW (10%) and L-glutamine (50-300 mg l^{-1}). All media were gelled with 2.2 g l^{-1} Gelrite. In all experiments explants were cultured in 250 ml conical flasks or disposal jam bottles (86 x 120 mm) containing 40 ml medium. The pH of the media was adjusted to 5.6 before autoclaving. All cultures were incubated at 24 ± 1°C, under the same light conditions as defined above.

Plantlet formation

In PLB proliferation medium, old PLBs developed into leafy structures from the base of which new PLBs emerged. These leafy structures or shoots were subcultured on MS medium with 2% sucrose, 2 g l^{-1} peptone, 10% CW and 1 g l^{-1} AC and gelled with 2.2 g l^{-1} Gelrite for plantlet formation with roots. Banana pulp (25-100 g l^{-1}; ripe banana, *Musa sapientum*, was peeled, pulp was made into a paste in a mortar with pestle, weighed and mixed with medium before autoclaving) was also tried to study its efficacy in differentiation and root growth.

Acclimatization and establishment of regenerated plantlets in the nursery

For acclimatization the rooted plantlets (between 50 and 70 mm in height) were taken out from the media and washed with tap water to remove the gel adhered to the roots. They were then implanted in a plastic basket containing three substrates: substrate A (coconut husk (20 mm^2); substrate B (coconut husk (20 mm^2) and charcoal (10 mm^3) (2:1)); substrate C (tree bark (10 mm^3) and charcoal (10 mm^3) (2:1)). To maintain high humidity the plantlets were treated with three periods of misting: 4 hrs (treatment 1), 6 hrs (treatment 2) and 8 hrs (treatment 3) intervals for 30 days. After acclimatization the plantlets were transplanted to 15 cm plastic pots perforated at the bottom and containing coconut husk (20 mm^2) and charcoal (10 mm^3) (2:1) and maintained under shade net at 30/25°C (day/night). Plants were watered every alternate day and fertilized with 6.5N-4.5P-19K solution at 10-day intervals.

Statistical treatment

Experiments were performed in a randomized design and all experiments were repeated three times. In *in vitro* culture each treatment had 15 replicates. The morphogenetic response of explants for PLB induction was evaluated after 12 weeks of culture. For PLB proliferation and plantlet regeneration, results were evaluated within 8 weeks of culture. For acclimatization 50 plants were taken for each treatment. Data were statistically analyzed and means were compared using Duncan's multiple range test (Duncan 1955).

RESULTS AND DISCUSSION

Induction of PLBs

In medium with BA or Kn alone less than 10% explants responded to induce 2-3 PLBs per explant (data not shown). Kn-NAA combinations responded to PLB induction in 15-26% explants depending on the concentration of Kn and NAA (data not shown). BA-NAA combinations increased the rate of PLB induction and the optimum concentration of BA and NAA for PLB induction was 1.5 and 0.5 mg l^{-1}, respectively, in which 65.5% of cultures produced a maximum of 8 PLBs per explant within 12 weeks (**Table 1**). The explants cultured directly on gelled medium gradually turned brown and ultimately dried but if they were first cultured in ½MS liquid medium with same components for one week and then subcultured on gelled MS medium PLBs were induced. The TCL explants showed small beaded structures on their surface after 4 weeks of subculture and they continued to develop into PLBs for an additional 8 weeks of culture (**Fig. 1A**). In previous experiments on *Cattleya*, a section of the meristem was used as the explant on MS medium with 0.5 to 1 mg l^{-1} IAA or NAA (Lindemann *et al*. 1970) or 0.1 mg l^{-1} NAA and 1.0 mg l^{-1} BA (Huang 1984) to initiate cultures. Superiority of liquid medium for initiation of cultures has been reported (Pierik 1987). Similarly, the use of NAA in combination with BA was needed for the in *in vitro* culture of *Aranda* (Lakshmanan *et al*. 1995), *Dendrobium* (Sinha *et al*. 2003) and *Phalaenopsis* (Sinha *et al*. 2006, 2007). In another experiment shoot organogenesis was induced from leaf explants of *Cattleya* cultured in MS medium with 1 mg l^{-1} Kn (Torres and Jimenez 2004). In the present study we optimized the BA-NAA combination, and obtained the highest number of PLBs in TCLs of *Cattleya* cultured on MS medium supplemented with 1.5 mg l^{-1} BA and 0.5 mg l^{-1} NAA along with 2% (w/v) sucrose, 10% CW, 2 g l^{-1} peptone and 1 gl^{-1} activated charcoal. Torres and Jimenez (2004) used 3% sucrose and 3.5 g l^{-1} activated charcoal in *Cattleya* culture. The positive effect of peptone was also reported in other orchid species culture (Alam *et al*. 2006; Sinha *et al*. 2006, 2007). It is documented that if coconut water is used with auxins it strongly induces cell division in tissues (Steward 1958). Kusumoto (1979a, 1979b) also reported that addition of coconut water to *Cattleya* culture medium enhanced proliferation of protocorms and stimulated shoot development. Coconut water contains 9-β-D-ribofuranosylzeatin, a cytokinin (Pierik 1987) and its presence (5-15% v/v) in the culture medium enhances the growth and proliferation of propagules, especially in orchid culture (Park *et al*. 2002; Sinha *et al*. 2003; Nayak *et al*. 2006) probably due to the additional amount of cytokinin. In the *in vitro* regeneration of orchids TCLs or thin cross sections of organs were also used as explants for *Aranda* (Lakshmanan *et al*. 1995), *Rhynchostylis gigantea* (Bui *et al*. 1999) and *Phalaenopsis amabilis* (Sinha *et al*. 2007) and it was reported that thin cross sections or TCLs of plant organs controlled morphogenesis more strictly than regular multi-tissue/organ explants (Teixeira da Silva *et al*. 2004).

Table 1 Effects of BA and NAA on PLB induction from thin cell layers of *Cattleya* after 12 weeks of culture on MS medium supplemented with 2% sucrose, 10% coconut water, 2 g l^{-1} peptone and 1 g l^{-1} activated charcoal.

Treatments (mg l^{-1})		Mean percentage of PLB-forming explants	Mean number of PLBs per explant
BA	NAA		
0	0	0	0
0.5	0.2	0	0
1.0	0.2	40.5 c	3.0 c
1.5	0.2	50.5 b	4.3 b
2.0	0.2	54.0 b	4.2 b
2.5	0.2	43.5 c	2.5 c
0.5	0.5	52.0 b	4.0 b
1.0	0.5	55.5 b	4.1 b
1.5	0.5	65.5 a	8.5 a
2.0	0.5	54.5 b	5.0 b
2.5	0.5	43.5 c	3.1 c

Mean values followed by the same letter are not significant at $P = 0.05$ by Duncan's multiple range test.

Fig. 1 PLB formation and regeneration of white flowered hybrid *Cattleya* from thin cell layer explants of young shoot. (**A**) PLBs formation from the explants cultured on MS medium with 1.5 mgl^{-1} BA + 0.5 mgl^{-1} NAA + 2% (w/v) sucrose + 10% (v/v) CW + 2 gl^{-1} peptone + 1 gl^{-1} AC for 12 week; (**B**) development of leafy shoots from PLBs clump cultured on MS medium with 2% (w/v) sucrose + 10% (v/v) CW + 2 gl^{-1} peptone + 150 mgl^{-1} L-glutamine + 1 gl^{-1} AC for 8 weeks; (**C**) development of plants with roots cultured on ½ MS containing 2% (w/v) sucrose + 2 gl^{-1} peptone + 10% (v/v) CW + 1 gl^{-1} AC for 8 weeks; (**D**) Plantlets with stout roots growing from leafy shoots cultured on ½ MS containing 2% (w/v) sucrose + 2 gl^{-1} peptone + 10% (v/v) CW + 1 gl^{-1} AC + 50 gl^{-1} banana pulp for 8 weeks. (**E**) Acclimatized plantlets.

Proliferation of PLBs

Regeneration of *Cattleya* was achieved through different experiments using different defined culture media. Solid and liquid MS media with 20% CW were used for proliferation of PLBs but for plantlet formation KC (Knudson 1946) medium was used (Arditti and Ernst 1993). Other researchers used MS medium for proliferation and differentiation of PLBs. KC (Knudson 1946), LM (Lindemann *et al*. 1970), and VW (Vacin and Went 1949) were also used to proliferate and differentiate PLBs, and it was observed that the survival percentage of the explants and multiplication of PLBs was high on MS medium. In the present experiment initially induced PLBs clumps (eight PLBs per clump) were subcultured for 8 weeks for the enhancement of PLBs and then dissected vertically into eight pieces. Each piece, having 5-6 PLBs, was subcultured on Gelrite-gelled MS medium in combination with 2% (w/v) sucrose + 2 g l^{-1} peptone + 1 g l^{-1} AC + 10% (v/v) CW and 150 mg l^{-1} L-glutamine. In this medium high-frequency proliferation (220 PLBs per explant) of PLBs was obtained within eight weeks of culture (**Table 2**).

Table 2 Effect of MS and ½MS media with/without coconut water and L-glutamine (along with 2% sucrose + 2 gl^{-1} peptone + 1 gl^{-1} activated charcoal) on PLB proliferation of *Cattleya* after 8 weeks of culture.

½MS			MS		
Treatments		**Mean**	**Treatments**		**Mean**
CW (%)	**L-glutamine (mgl^{-1})**	**number of PLBs per explant**	**CW (%)**	**L-glutamine (mgl^{-1})**	**number of PLBs per explant**
0	0	11.6 f	0	0	21.3 g
10	0	14.7 e	10	0	43.4 f
10	50	20.0 d	10	50	72.4 e
10	100	27.5 c	10	100	92.5 d
10	150	46.5 a	10	150	220.2 a
10	200	34.5 b	10	200	163.5 b
10	250	20.2 d	10	250	120.5 c
10	300	15.2 e	10	300	98.4 d
0	50	10.6 f	0	50	31.5 g
0	100	21.2 d	0	100	66.5 e
0	150	20.3 d	0	150	102.3 d
0	200	10.8 f	0	200	65.6 e
0	250	10.2 f	0	250	60.3 e
0	300	8.2 f	0	300	18.6 g

Mean values followed by the same letter are not significant at $P = 0.05$ by Duncan's multiple range test.

Lindemann *et al*. (1970) used glutamic acid (or glutamine), asparagine, peptone, or casein hydrolysate in liquid MS medium and subsequently the PLBs were subcultured in gelled KC (Knudson 1946) medium. In our study, the effect of L-glutamine on protocorm proliferation was evaluated and the highest number of PLBs per culture was obtained on media containing 150 mg l^{-1} L-glutamine and 220 PLBs developed from a single clump of 5-6 PLBs. This high-frequency regeneration of PLBs was possible probably due to the synergistic effect of the organic compounds present in CW and L-glutamine. Moreover, subculture of clumps of PLBs is much easier and efficient in comparison with subculture of individual PLB sections. The positive effects of CW and L-glutamine in culture medium on multiplication rates of somatic embryos in other plant species were reported (Kako 1973; Pierik 1987; Alam *et al*. 2006; Sinha *et al*. 2006). After a further four weeks of culture (in the same medium) the PLBs enlarged with leafy shoots (**Fig. 1B**). The leafy shoots were subcultured for conversion into plantlets and the non-leafy bodies were subcultured on fresh PLB proliferation medium with the same constituents for their development into leafy shoots and further multiplication. Thus, in each subculture profuse PLBs as well as leafy shoots were produced. Within the first 33 weeks after initiation of culture 1760 leafy shoots as well as a huge amount of PLBs were obtained from a single explant section or TCL. Within the next 32 weeks 500,000 leafy shoots were produced. The procedure was repeated and it has continued for several years now without the deterioration of protocorm regeneration potential.

Regeneration of plantlets

The leafy shoots were subcultured on ½MS medium with 2 g l^{-1} peptone, 2% (w/v) sucrose, 10% CW and 1 g l^{-1} AC, upon which 100% explants developed into plantlets with roots (**Fig. 1C**) within 8 weeks. Teixeira da Silva and Tanaka (2006), through histological and cytological analyses, proved for the first time that the term somatic embryo could be used synonymously with the term PLB, only for orchids, explaining why both roots and shoots would inevitably form from such cultures. The addition of 50 g l^{-1} banana pulp in the medium enhanced the growth and number of roots (**Table 3; Fig. 1D**) as it is especially promotive for growth in orchid culture (Pierik 1987). Other researchers obtained plantlets of *Cattleya* with root induction in modified KC medium (Lindemann *et al*. 1970), modified MS

Table 3 Effect of banana pulp on rooting of regenerated shoots of *Cattleya* cultured on half strength MS medium with 2% sucrose + 10% (v/v) coconut water + 2 gl^{-1} peptone + 1gl^{-1} activated charcoal. Data were recorded up to 8 weeks after inoculation.

Weeks after culture	Banana pulp (gl^{-1})	% Shoots rooted	*Mean number of root ± SE	Mean length of root ± SE
2	0	0	0	0
	25	0	0	0
	50	20.28	2.5 ± 0.04 d	21.3 ± 1.2
	75	32.56	2.6 ± 0.05 d	19.3 ± 1.0
	100	12.68	2.2 ± 0.07 d	15.7 ± 0.8
4	0	28.82	2.8 ± 0.05 d	22.3 ± 1.5
	25	42.37	3.0 ± 0.06 cd	28.6 ± 2.0
	50	60.65	3.5 ± 0.08 c	35 9 ± 2.0
	75	41.28	2.9 ± 0.09 cd	23 6 ± 1.8
	100	31.50	2.4 ± 0.05 d	19.9 ± 1.3
6	0	51.83	2.5 ± 0.04 d	32.6 ± 2.3
	25	67.25	3.4 ± 0.06 c	30.8 ± 1.9
	50	100	4.2 ± 0.09 b	45.8 ± 2.5
	75	50.55	3.8 ± 0.05 c	25.8 ± 1.5
	100	32.38	3.3 ± 0.07 c	20.5 ± 1.3
8	0	100	3.5 ± 0.08 c	40.9 ± 2.4
	25	100	4.4 ± 0.07 b	45.6 ± 2.8
	50	100	5.2 ± 0.09 a	48.9 ± 2.3
	75	68.25	4.5 ± 0.08 b	28 7 ± 1.8
	100	42.64	3.4 ± 0.06 c	26.7 ± 1.4

15 cultures were taken for each treatment and the experiments were repeated three times.

*Mean *values* followed by the same *letter* are not significant at $P = 0.05$ by Duncan's multiple range test.

medium with 0.3 mg l^{-1} NAA and 30 mg l^{-1} adenine sulphate (Huang 1984) and half strength MS medium supplemented with 3.5 g l^{-1} AC and 10% CW (Torres and Jimenez 2004). Kusumoto (1979a, 1979b) also obtained positive results by adding banana extract to *Cattleya* culture. In the present experiment identical leafy shoots were separated from the PLB proliferation medium and subcultured for their conversion into plantlets for obtaining a large number of identical plantlets rapidly.

Within the first 41 weeks after initiation of culture 1760 plantlets as well as a huge amount of PLBs were achieved from a single explant section. Repeating the subculture of PLBs on proliferation medium and culturing leafy shoots on plantlet regeneration medium, could produce 500,000 plantlets every 32 weeks.

Acclimatization

The survival of regenerated plantlets during acclimatization did not depend significantly on the substrate used in the culture pots (**Table 4**). The air humidity had the most significant effect on the survival of the plantlets. Humidity maintained by misting at 6-h intervals was found to be optimum in which 80-85% plantlets survived vigorously. If the misting interval was shorter (4 hrs), the survival rate was only 50-55% due to excessive moisture. When they were maintained by misting at 8-hr intervals transplanted plantlets gradually declined due to withering and only 40-45% plantlets were successfully acclimatized. After acclimatization the plantlets were transplanted to 15 cm plastic pots perforated at the bottom and containing coconut husk (20 mm^2) and charcoal (10 mm^3) (2:1) and they were maintained under shade net at 30/25°C (day/night). No mortality occurred after acclimatization and growth performance was normal without any morphological abnormalities.

Table 4 Acclimatization (%) of *in vitro* regenerated plants of *Cattleya* treated with different types of misting in different substrates. Data were taken after 4 weeks of culture.

Variants of acclimatization		%* of acclimatized
Substrates	**Humidity**	**plants (Mean ± SE)**
	Treatment 1	50.8 ± 6.5 b
A	Treatment 2	80.8 ± 5.5 a
	Treatment 3	45.5 ± 5.3 c
	Treatment 1	52.8 ± 4.5 b
B	Treatment 2	85.3 ± 6.5 a
	Treatment 3	42.5 ± 5.4 c
	Treatment 1	50.8 ± 5.2 b
C	Treatment 2	84.6 ± 5.8 a
	Treatment 3	44.5 ± 4.1 c

*50 plants were taken for each treatment and the experiments were repeated three times.

Mean values followed by the same letter are not significant at $P = 0.05$ by Duncan's multiple range test.

As in the present study plants were directly regenerated through PLBs formation from explants without the intervention of callus, and the growth regulators (BA and NAA) were used in small doses only in PLB induction medium, there was no risk of somaclonal variation. In *in vitro* culture the excessive use of growth regulators, and especially during the intermediary callus phase, is undesirable (Prakash *et al*. 1996) as those are thought to be the main causes of variation in plants cultured *in vitro* (George and Sherrington 1984); in the present study it was avoided. The present study also reveals the advantage of the tTCL system that produce a high frequency of PLBs and consequently shoot regeneration and reduce the time interval required (Lakshmanan *et al*. 1995).

CONCLUSIONS

In conclusion, an efficient protocol for reproducible high frequency regeneration of white cultivar of *Cattleya* hybrid in a simple culture medium and with short culture period was established. We report here the induction of PLBs of *Cattleya* from TCLs of young shoots of mature plants for the first time in MS medium with 1.5 mg l^{-1} BA and 0.5 mg l^{-1} NAA and optimized the high-frequency PLB proliferation medium (MS supplemented with 2 g l^{-1} peptone + 2% (w/v) sucrose + 10% (v/v) CW + 150 mg l^{-1} L-glutamine + 1 g l^{-1} AC) and method (culture of a section of PLB clumps, instead of individual PLB sections) and conversion of leafy shoots into plantlets in simple ½MS medium. Thus, through repeated subculture of PLBs clumps which results in leafy shoots, in turn, subculture to plantlet regeneration medium leads to the harvest of regenerated plantlets, and continuous high-frequency production of plants could be maintained.

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